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CRDEC-TR-88153

MIPAFOX AS A SUBSTRATE FOR RANGIA-DFPase

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August 1988

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REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S) CRDEC-TR-88153			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION CRDEC		6b. OFFICE SYMBOL (if applicable) SMCCR-RST-E		7a. NAME OF MONITORING ORGANIZATION	
6c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423			7b. ADDRESS (City, State, and ZIP Code)		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION CRDEC		8b. OFFICE SYMBOL (if applicable) SMCCR-RST-E		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.
				1C161102	A71A
11. TITLE (Include Security Classification) Mipafox as a Substrate for Rangia-DFPase					
12. PERSONAL AUTHOR(S) Chester, Nancy A.; Anderson, Robert S., Ph.D.; and Landis, Wayne G., Ph.D.					
13a. TYPE OF REPORT Technical		13b. TIME COVERED FROM 86 Sep to 86 Oct		14. DATE OF REPORT (Year, Month, Day) 1988 August	
15. PAGE COUNT 16					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP			
06	11		Mipafox DFPase		
15	06	03	Rangia-DFPase DFP		
			Substrate		
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
<p>The effect of mipafox (N,N'-diisopropylphosphordiamidofluoridate) on the DFPase activity of clam digestive gland was investigated. An increasing trend in hydrolysis rates resulted where mipafox activity < DFP activity < DFP with mipafox activity. Mipafox is a known inhibitor of DFPase from hog's kidney and <i>Tetrahymena thermophila</i> but does not inhibit "squid-type" DFPase. The observed additive effect of DFP with mipafox hydrolysis rates appears, in this case, to be a unique characteristic of Rangia-extract.</p> <p style="text-align: right;">Amides, Fluorine Compounds, Organic Fluorine compounds</p>					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION UNCLASSIFIED		
22a. NAME OF RESPONSIBLE INDIVIDUAL SANDRA J. JOHNSON			22b. TELEPHONE (Include Area Code) (301) 671-2914		22c. OFFICE SYMBOL SMCCR-SPS-T

DD FORM 1473, 84 MAR

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PREFACE

The work described in this report was authorized under Project No. 1C161102A71A, Research in CW/CB Defense. This work was started in September 1986 and completed in October 1986.

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MIPAFOX AS A SUBSTRATE FOR RANGIA-DFPase

1. INTRODUCTION

Enzymes capable of hydrolyzing 0,0-diisopropylphosphorofluoridate (DFP), and related acetylcholinesterase inhibitors such as 0-1,2,2-trimethylpropylmethylphosphonofluoridate (soman), and 3,3-dimethylbutylmethylphosphonofluoridate (Dimebu) have been reported in the tissues of many animals.¹⁻⁴ Previously, two categories of DFP hydrolase enzymes, or DFPases, were partially characterized. "Squid-type" DFPase hydrolyzes DFP faster than soman; is stable; has a molecular weight of approximately 26,000; is inhibited by a manganous ion; and is present in optic ganglia, giant nerve axon, hepatopancreas, and the salivary gland of cephalopods. "Mazur-type" DFPase is stimulated by a manganous ion, hydrolyzes soman faster than DFP, is dimeric with a molecular weight of approximately 62,000, and is unstable. Activities resembling Mazur-type are found in a hog's kidney, Escherichia coli, mammalian tissues, the protozoan Tetrahymena thermophila, and the clam, Rangia cuneata.^{2,3} It is important to note, however, that several DFPase sources have been shown to consist of more than one DFPase.⁵⁻⁷

The use of mipafox (N,N'-diisopropylphosphordiamidofluoridate) has been introduced to DFP hydrolysis studies as a tool for further enzyme identification and characterization. If soman or DFP is used as a substrate, mipafox is a potent, reversible, competitive inhibitor of "Mazur-type" but not "squid-type" DFPase. Based upon these findings, the study also confirmed that various tissues, particularly Escherichia coli and squid, are mixtures of both DFPases.⁶ The following study describes a series of mipafox and DFP assays that were performed using extracts from the digestive gland of Rangia cuneata.

2. METHODS AND MATERIALS

2.1 Tissue Preparation.

Mature estuarine clams (Rangia cuneata) were collected in sediment samples from the Chesapeake Bay near the Aberdeen Proving Ground and held in ambient water at 2 °C for several hours prior to processing. Homogenates, 33% clam tissue by weight in Hanks balanced salt solution (HBSS) (DIFCO, Detroit, MI), were prepared from digestive glands pooled from 30-50

individuals and stored at 4 °C. Prior to testing, the tissue was diluted 1:4 with HBSS (clam 1) and Hoskin's buffer (400 mM KCl, 50 mM NaCl, and 5 mM 1,3-bis[tris hydroxymethyl methylamino] propane [bis-tris-propane] in glass distilled water, ph 7.2) (clam 2). The material was centrifuged at 1500 rpm for 20 min yielding supernatant used in the assay. One aliquot of each preparation was subjected to 70 °C for 30 min.

2.2 Enzyme Assay.

Hoskin's buffer was used in the activity assays, and all chemicals were reagent grade. Three substrate solutions were individually tested for activity: 3.0×10^{-3} M DFP, 3.0×10^{-3} DFP with 3.0×10^{-3} mipafox, and 3.0×10^{-3} M mipafox were added to a 5-mL disposable beaker equipped with a magnetic stirrer. Substrate hydrolysis was quantified using a fluoride electrode attached to an Orion 901 microprocessor ionanalyzer that recorded fluoride concentrations at 1-min intervals. After recording spontaneous hydrolysis, enzyme-mediated hydrolysis was measured following the addition of 100 μ L of extract. Protein concentrations of tissues were measured using the Biorad system.⁸ Reaction rates were calculated using the Apple-compatible program, DFPASE2 (see Appendix).

3. RESULTS

Figure 1 shows DFP and mipafox hydrolysis rates (average of five replicates) using clam 1 and clam 2 digestive gland. Mipafox hydrolysis rates appear to be lowest (Table) and are an average of 59.4% and 89.2% lower than the DFP hydrolysis rates for clam 1 and clam 2, respectively. DFP with mipafox activities are the highest (Table) with average rates of 117.3% and 17.2% of the DFP rates for clam 1 and clam 2. Overall, these values present a trend in hydrolysis rates where mipafox < DFP < DFP with mipafox. The denaturization of tissue at 70 °C for 30 min was found to destroy activity in both clam preparations.

Table. Hydrolysis Rates in μ moles/g Protein/Min. (Mipafox rates are an average of 59.4% and 89.2% lower than DFP rates, while DFP with mipafox activities are an average of 117.3% and 17.2% of DFP rates.)

	Mipafox	DFP	DFP with mipafox
Clam 1	17.6 (.564)*	42.4 (1.50)	52.5 (5.56)
Clam 2	3.98 (.530)	37.0 (3.31)	39.4 (3.31)

*Standard Error

4. DISCUSSION

The use of mipafox in this study has further characterized the DFPase activity of extract from the Rangia-digestive gland. The trend of increasing activities from mipafox hydrolysis, to DFP hydrolysis, to DFP with mipafox hydrolysis is unique. These hydrolysis rates appear to be a characteristic of the digestive gland of a clam, suggesting an additive effect of mipafox and DFP hydrolysis. A similar assay with squid-type DFPase results in no inhibition of DFP hydrolysis by mipafox.⁶ Assays performed with Tetrahymena thermophila and hog kidney, which predominantly contain "Mazur-type" DFPase,⁵ show 79.0% and 83.9% inhibition of DFP hydrolysis by mipafox (Figure 2). Although the activity in the clam digestive gland has previously been determined as predominantly "Mazur-type"¹, it is possible that the combination hydrolysis rates are the result of a DFPase and a mipafox-hydrolyzing enzyme. Studies are currently underway that will investigate clam enzyme kinetics, including a study in which purified (via column separation) clam-DFPase will be assayed with mipafox and DFP.

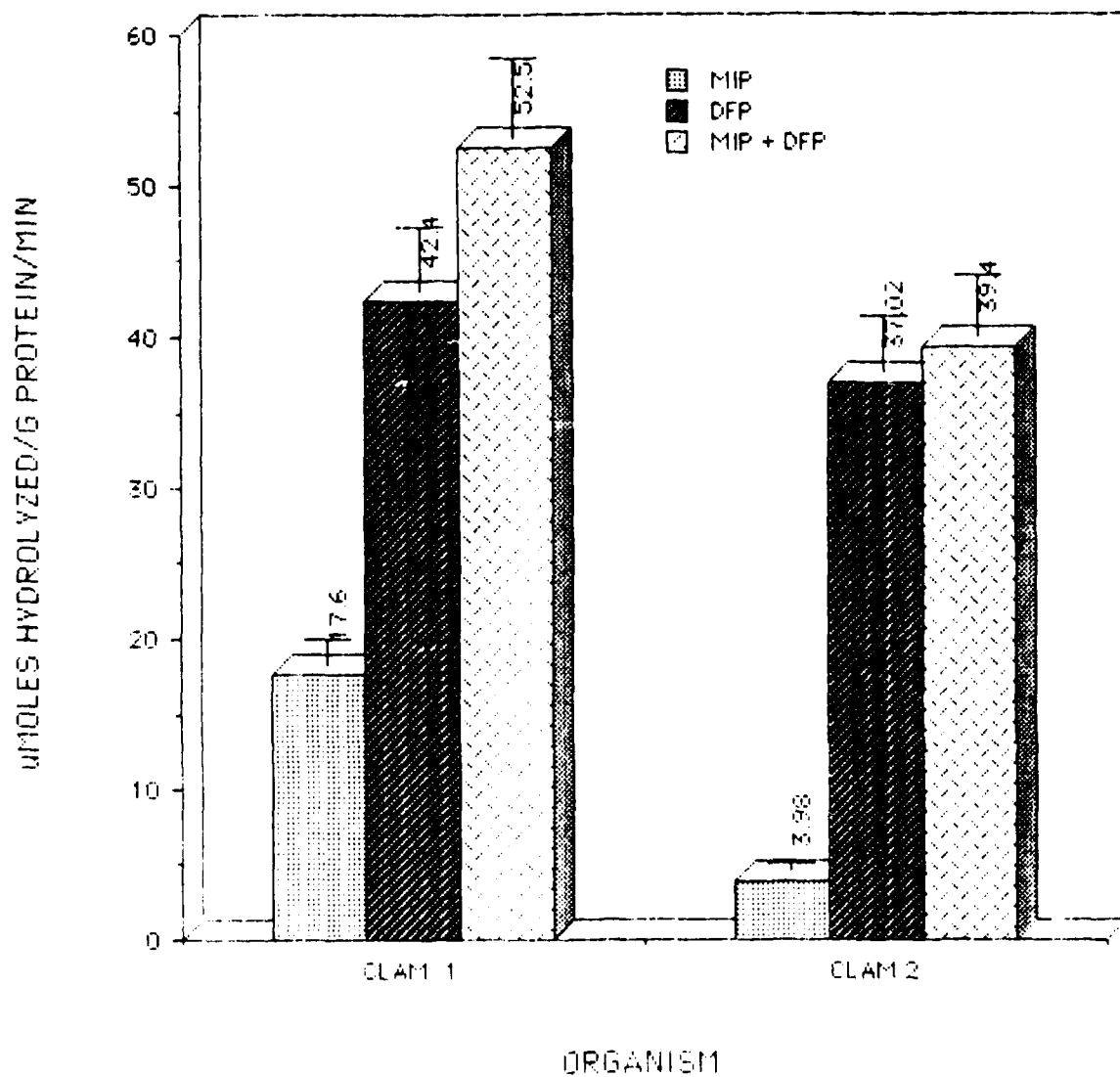


Figure 1. Hydrolysis of Mipafox and DFP by Clam-Digestive Gland. (Clam 1 and clam 2 hydrolysis rates represent a trend of increasing activities. Mipafox values are the lowest of all groups, while DFP with mipafox rates appear to be the sum of DFP and mipafox hydrolysis.)

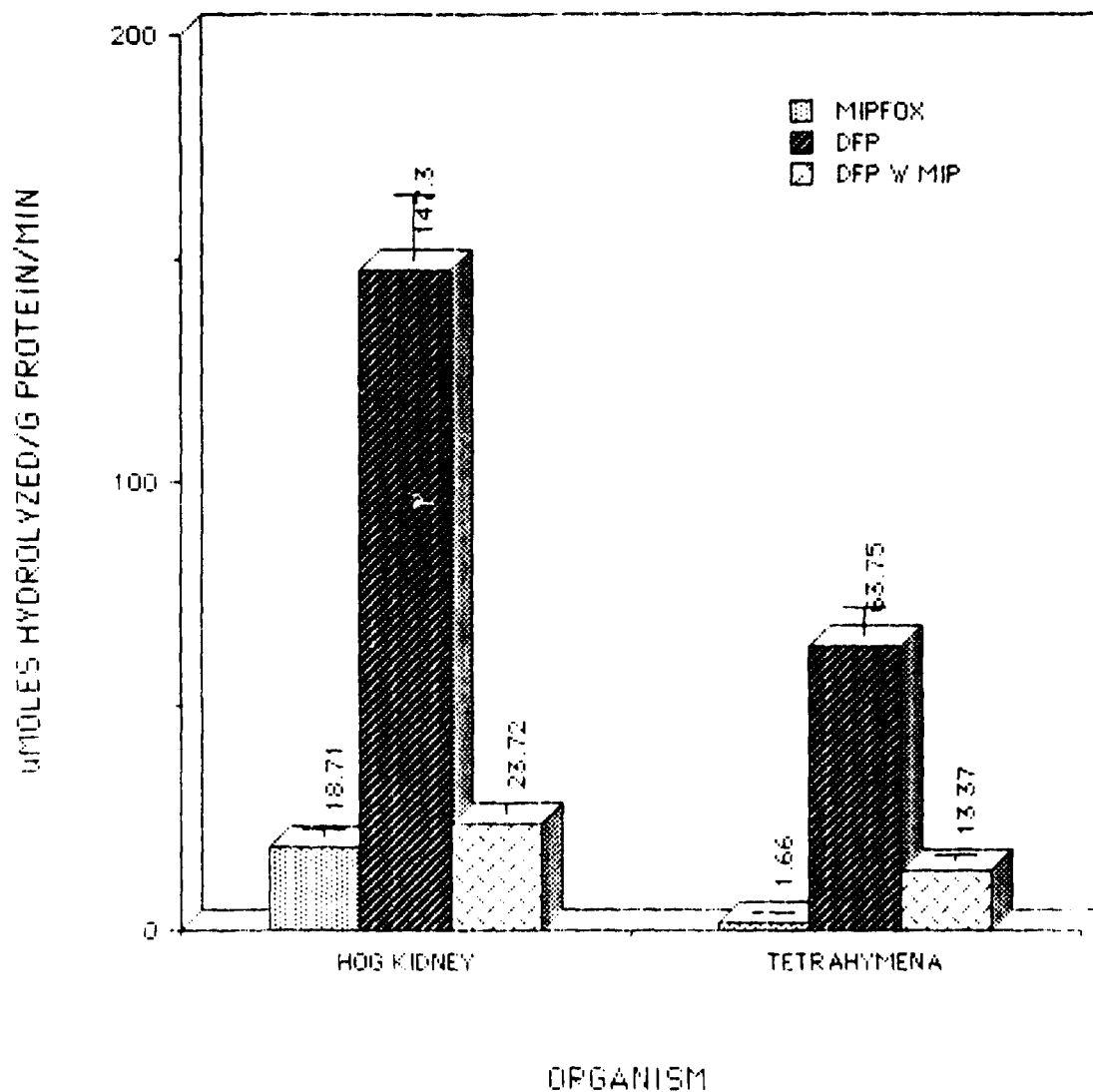


Figure 2. Hydrolysis of Mipafox and DFP by Tetrahymena thermophila and Hog Kidney. (DFP hydrolysis is inhibited 79.0% in Tetrahymena thermophila and 83.9% in hog kidney by mipafox.)

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APPENDIX

APPLE DFPASE2 PROGRAM

```

1  PR# 3
2  INVERSE
5  PRINT "*****DFPASE PROGRAM 2 *****"
   *****
10 PRINT "PROGRAM FOR CALCULATION OF ENZYMATIC HYDROLYSIS RATES OF "
20 PRINT "ORGANOFLUOROPHOSPHATES BY DFPASE AS MEASURED BY FLUORIDE
   EVOLUTION"
30 PRINT "INPUTS REQUIRED ARE: TIME OF REACTION IN MINUTES, REACTION
   VOL IN ML"
40 PRINT "VOLUME OF ENZYMATIC PREP IN ML, PROTEIN CONC. IN MG/ML, THE "
50 PRINT "STARTING AND FINISHING CONC. OF FLUORIDE IN uM, OR THE DELTA
   IN uM"
55 PRINT " AND THE SPONTANEOUS HYDROLYSIS RATE IN uM/MIN"
60 NORMAL
61 PRINT " INPUT SPONTANEOUS HYDROLYSIS"
62 INPUT S
70 PRINT "INPUT TIME, REACTION VOLUME"
80 INPUT T,V
90 PRINT "INPUT VOL. ENZYMATIC PREP, PROTEIN CONC."
100 INPUT E,P
105 S1 = S * T
110 T1 = 60 / T
120 V1 = 1000 / V
130 E1 = 1 / E
131 PRINT "IF INPUT IS START AND FINISH CONC. KEY 0, IF DIFFERENCE KEY
   1"
132 INPUT W
133 IF W = 0 THEN GOTO 140
134 IF W = 1 THEN GOTO 135
135 PRINT "INPUT DELTA uM"
136 INPUT CT
137 GOTO 161
140 PRINT "INPUT START FLUORIDE, FINISH FLUORIDE"
150 INPUT C1, C2
160 CT = C2 - C1
161 CC = CT - S1
170 R1 = CC * ((E1 * T1) / V1)
171 PRINT "RUN NUMBER ="
172 INPUT W
174 PR# 1
175 PRINT "*****"
   *****
178 PRINT "RUN NUMBER=";W;"SPONTANEOUS HYDROLYSIS RATE=";S
179 PRINT "TIME=";T;"VOL=";V;"ENZ PREP=";E;"PROTEIN=";P;"uM=";CC
180 PRINT R1;"uMOLES HYDROLYZED /ML/HR"
190 R2 = R1 / 60
200 PRINT R2;"uMOLES HYDROLYZED /ML/MIN"
210 IF P = 0 THEN GOTO 265
220 P1 = 1000 / P
230 R3 = R1 * P1
240 PRINT R3;"uMOLES HYDROLYZED /G PROTEIN/HR"

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250  R4 = R3 / 60
260  PRINT R4;"uMOLES HYDROLYZED /G PROTEIN/MIN"
265  PRINT  "*****"
*****
266  PR# 0
267  PR# 3
270  PRINT "KEY 1 IF ONLY THE START AND FINISH CONC. CHANGE"
280  PRINT "KEY 2 IF TIME, VOL, PROTEIN CONC., OR VOL. ENZYME PREP
CHANGES"
285  PRINT "KEY 3 IF THE RATE OF SPONTANEOUS HYDROLYSIS ALSO CHANGES"
290  PRINT "KEY 4 IF YOU ARE FINISHED"
300  INPUT Z
310  IF Z = 1 THEN GOTO 131
320  IF Z = 2 THEN GOTO 70
330  IF Z = 3 THEN GOTO 61
335  IF Z = 4 THEN GOTO 390
390  END

```